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Introduction

Estrogens are important regulators of growth and differentiation in the normal mammary gland and are also important in the development and progression of breast carcinoma (1). Estrogens regulate gene expression via estrogen receptor (ER) and, because approximately two-thirds of all breast cancers are ER+ at the time of diagnosis, the expression of the receptor has important implications for their biology and therapy (2).

ER binds to the estrogen response element (ERE) found in the promoters of estrogen-regulated genes and activates their transcription (3). However the details of this activation, the role of cofactors, the number and name of the gene targets, and the cross-talk between other signaling pathways are far from fully known or understood.

Recently, it has been shown that there are two different ER isoforms encoded by two different genes: ER α and ER β (4). Both isoforms are highly similar in the DNA-binding domain and, to a lesser degree, in the ligand-binding domain (5).

Several ER-associated proteins, coactivators and corepressors, have been identified that are of importance in regulating the ER interaction with the basal transcription machinery (6). Between the coactivators, a group of proteins of approximately 160 KDa molecular mass were among the first factors identified that interact with nuclear receptors in a highly ligand-dependent manner (7, 8). Expression cloning and yeast two-hybrid screening approaches led to the identification of three related genes that encode these factors, referred to as SRC-1/NCoA-1, TIF2/GRIP-1/NCoA2, and p/CIP/AIB1/ACTR/RAC3/TRAM-1 (6). Several lines of evidence support the idea that p160 factors play important roles as nuclear receptor coactivators, in part by recruiting CBP/p300 and arginine methyltransferase (9), although the extent of their role is not proven. Interestingly, p/CIP was identified and shown to be highly amplified in 10% primary breast cancers and overexpressed in 64% breast cancers examined (10).

On the other hand, in the absence of ligand, several nuclear receptors suppress basal gene transcription by recruiting corepressors (6). Nuclear receptor corepressor (NCoR) (11, 12) and silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) (13) have been intensively studied in the context of the unliganded thyroid hormone receptor (TR) and retinoic acid receptor (RAR). The amino acid sequences of NCoR and SMRT show approximately 40% identity. In contrast to other nuclear receptors, suppression of ER-mediated basal transcription by corepressors has not been observed. However, there is evidence that ER interacts with them *in vitro* and NCoR coimmunoprecipitates with ER weakly without ligand and more strongly in the presence of tamoxifen (14).

Moreover, it has been demonstrated that, upon the binding of the anti-estrogen TOT, ER binds to corepressors effectively. However, in the absence of N-CoR, TOT is converted from an antagonist to an agonist with regards to ER function (14). N-CoR expression is upregulated during late pregnancy when proliferation of mammary epithelial cells (MEC) slows and the alveoli begin to functionally differentiate by producing milk proteins (15). This expression profile is consistent with NCoR's function as a repressor of ER-mediated transcription since estrogen is required for proliferation of MEC, the burst of which occurs between 2-6 days of pregnancy. The ability of NCoR to function as a repressor of ER-mediated transactivation suggests that NCoR may play an important role not only during

normal mammary gland biology, but also in the process of breast tumorigenesis and the acquired resistance to tamoxifen treatment.

Tamoxifen is a selective estrogen receptor modulator (SERM) that competes with estrogen for binding to the estrogen receptor. In different tissues and in different animal species, tamoxifen, like other SERMs, may act as an agonist or antagonist of estrogen. In humans, tamoxifen acts as an estrogen antagonist in breast tissue, inhibiting the growth of estrogen-dependent breast tumors. In other tissues such as endometrium or bone, and in the serum lipoprotein fractions, tamoxifen acts as an estrogen agonist in that it induces endometrial proliferation, preserves bone mass in postmenopausal women, and lowers LDL cholesterol (16). Tamoxifen has been shown to reduce the incidence of breast cancer by more than 40% (17, 18).

Body

Over the initial eight months of support under the DoD Breast Cancer Research Award, I have made significant progress on several specific aims of the initial statement of work, and I have elucidated a novel intriguing regulatory event with implications for estrogen receptor resistance in breast cancer. Initially, the starting grant period was May 2001, but it was delayed to October 2001. Therefore, this report is about the period between October 2001 and June 2002.

One of the initial goals was centered about defining potential roles of p/CIP protein in the biology of mammary gland development and tumor formation. To this end, I generated a transgenic targeting vector with the p/CIP coding sequence under 7 Kbp of the whey acidic protein (WAP) promoter (details described in the Research Design and Methods of the initial proposal). The transcription unit was tested in MCF-7 breast cancer cells and proven to robustly express p/CIP transcript and protein. That construct was then microinjected into male pro-nucleus of fertilized eggs from BL6 mice, and transgenic mice were screened by Southern blot analysis for detecting the presence of the integrated vector. Three lines were selected for breeding, and all of them show to over-express p/CIP in mammary gland when tested by Northern and western blot analysis. These mice will be bred and, over the next months, will be analyzed the breast development, the response to pregnancy, lactating and involuting, and the susceptibility to tumor formation, like described in task II (initial proposal). When colony will be appropriately expanded, breeding to p/CIP and N-CoR gene deleted mice will be mutated as described under task I (initial proposal).

During the course of the initial eight months, also we have done new and very special interesting observations for further interpretation. The first related to the role of inflammation in estrogen receptor antagonist effects of target gene expression. In MCF-7 breast cancer cells, tamoxifen (4-OHT) antagonizes the activating effects of 17β -estradiol (E_2) over $ER\alpha$. We have observed that this repressing effect is by recruiting N-CoR to $ER\alpha$. In recent experiments, we have observed that inflammatory cytokines, such as interleukin-1 ($IL-1\beta$), can repress the antagonist effect of tamoxifen on $ER\alpha$, causing fully activation of specific ER target genes. In parallel, we have evidences of the existence of a novel N-CoR complex composed by N-CoR, the histone deacetylase HDAC3, and a component of a $NF-\kappa\beta$

regulating complex, called TAB2. Unexpectedly, cell treatment with IL-1 β causes nuclear export of the new N-CoR/TAB2/HDAC3-containing complex to the cytoplasm. Our results indicate that the molecular basis of this IL-1-dependent nuclear export is most likely to represent a MEKK1-dependent phosphorylation of TAB2 in the nucleus, putatively causing an allosteric alteration of the protein that exposes the TAB2 nuclear export signal (NES) for exporting the whole N-CoR complex to the cytoplasm. That shuttling of this complex counterposes the repressing effect of tamoxifen over ER α -target genes. To explore with more detail the specificity of this action, I propose to use a new approach that combines the immunoprecipitation of specific target DNA fragments bound to a specific protein with the microarray analysis (methodology called ChIP-chip or genome wide location analysis) (19, 20).

In these experiments, MCF-7 cells will be grown to 95% confluence in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% charcoal-dextran-stripped fetal bovine serum for at least 3 days (21). Cell cultures will be treated with IL-1 β for 2 hours, and then with tamoxifen or estradiol for 1 hour. After rinsing with PBS buffer, cells will be cross linked during 20 minutes at room temperature with a PBS-1% formaldehyde solution. Cross linked cells will be harvested and kept to 30°C during 15 minutes in a Tris-DTT solution for stopping the cross linking reaction. Cellular proteins will be extracted and chromatin will be fragmented by sonication. Typically, fragments between 300 bp and 800 bp will be obtained with soft sonication. Specific proteins will be immunoprecipitated using specific antibodies: anti-ER α , anti-TAB2, anti-HDAC3 and anti-N-CoR. After reversal the cross links and DNA purification, DNA molecules will be amplified by ligation-mediated polymerase chain reaction (LM-PCR) and subsequently will be labeled with the Cy5 fluorophore by random priming (22). For purposes of normalization, we will perform LM-PCR on not enriched by immunoprecipitation sonicated genomic DNA and then, that amplified sample will be labeled with a second fluorescent dye, Cy3. ChIP-enriched and nonenriched pools of DNA will be mixed with Cot-1 DNA to suppress annealing of repetitive sequences and will be hybridized under stringent conditions to the 1.5 Kb DNA microarray.

Dr. Bing Ren's laboratory located in the Ludwig Institute (UCSD) has developed a promoter DNA array (referred to as 1.5 Kb array) that contains polymerase chain reaction (PCR) products spanning the proximal promoters of 1444 human genes, from which approximately 1200 show regulated expression during cell cycle and approximately 200 genes don't show apparent link to the cell cycle (22). The array is composed by PCR amplified regions that span around 700 bp upstream and 200 bp downstream of putative transcription start sites. These last based on algorithms that predict DNA-binding motifs within promoters.

Those studies would open important and amazing new aspects in the understanding of ER α and cofactors action, breast cancer, and tamoxifen resistance. Basically because the use of the ChIP-chip methodology will allow to know new direct ER α -target genes and to know when the repressing complex N-CoR/TAB2/HDAC3 is recruited to repress the transcription of specific genes in response to tamoxifen and IL-1 β treatments.

A parallel issue is that diverse evidence suggests a role for Wnt signaling in mammary gland development and tumorigenesis (23). One of them comes from LEF-1^{-/-}/LEF-1⁻ mice, which arrest tooth and mammary gland development at the "bud stage" (24). This is a stage of development when locally thickened epithelium invaginates into underlying condensed mesenchyme to form a characteristic bud structure, from which the mammary ductal system will develop (25). LEF-1/TCF is a family of transcriptional factors that activates the gene

expression when associated in a complex with β -catenin (23). This last is a coactivator protein that is shuttled to the nucleus when an external Wnt signal is recognized at the cellular membrane level by specific receptors and co-receptors, and the signal is transduced to repress the β -catenin cytoplasmic degradation pathways and Wnt signaling pathway, been ER α /estradiol pathway one of them (26, 27).

To know at molecular level if β -catenin/LEF-1/TCF complex or the coactivator β -catenin alone associates with ER α , I propose to purify multiprotein complexes of β -catenin in MCF-7 cells. I will construct a stable cell line expressing a fusion protein of a N-terminus mutated β -catenin and a C-terminus TAP (Tandem Affinity Purification) tag. This tag consists of a calmodulin-binding protein (CBP) and a protein A tags separated by a TEV protease cleavage site (28). The mutated β -catenin form is an N-terminal deleted protein that is not cytoplasmic regulated and, therefore, is directly sent to the nucleus (29). Nuclear extracts from the β -catenin-TAP expressing cells will be loaded in an IgG-column that specifically retains protein A tag. After extensively washes, TEV protease digestion will allow to elute β -catenin (with their associated proteins) from the column, and then the elution will be loaded in a calmodulin column in the presence of Ca⁺². Under these conditions, CBP is retained by calmodulin. After extensively washes, addition of EDTA will allow to elute β -catenin multiprotein complexes from the column, and using mass-spectrometry techniques, the specific eluted polypeptides will be identified.

Key research accomplishments

- Generating of a transgenic vector that express full-length p/CIP protein under the control of a mammary gland specific promoter.
- This vector is able to express the protein in transient transfection experiments using MCF7 cells.
- The construct has been microinjected to generate a transgenic mouse.
- Three selected lines over-expressed p/CIP in mammary gland have been established.

Conclusions

- The construction of a breast specific p/CIP-overexpressing transgenic line was one the main important aspects of the initial project, and its performing will allow to continue the tasks initially proposed.
- New interesting observations have been obtained in Dr. Rosenfeld lab that open new lines of study around the role of coactivators and corepressors in breast development and receptor-dependent tumorigenesis, specially in tamoxifen resistance. This allow me to pursue new experiments to perform during the next year that will be a significant progress in the understanding of the role of ER coactivators and corepressors in the initiation and progression of breast cancer as well as in susceptibility or resistance of breast cancer to tamoxifen treatment. Basically, these are the use of a new and extremely powerful

methodology, to identify new ER/cofactor gene targets and to determine the presence of ER/cofactors on them depending of the cellular conditions (tamoxifen, estradiol and I β treatments).

- In parallel, recent studies show a putative relation between Wnt signaling and ER/estradiol pathways, suggesting a cross talk in breast development and cancer. The purification of the classical coactivator of the Wnt pathway, β -catenin, and the associated proteins using nuclear extracts from stable-transfected MCF-7 cells, will help to understand the level of this relation.

- The use of the new proposed technologies and the new approaches will provide me a very useful training in the area of breast cancer biology.

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